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Incr ased Synthesis and Degradation of DNA Topoisomeras I during the Initial Phase of Human T Lymphocyte Prolif ration*

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The regulation of DNA topoisomerase I was studied in human T lymphocytes following phytohemagglutinin (PHA) stimulation. As T lymphocytes began to enter the S phase 24 h after stimulation, there was a rapid increase in DNA topoisomerase I mRNA. The level of DNA topoisomerase I mRNA increased continuously over the next 18 h and peaked (>50-fold increase) 42 h after stimulation with PHA. A concomitant increase in DNA topoisomerase I protein was also observed. However, the maximal increase in DNA topoisomerase I protein was only 6-fold. To explain the quantitative difference between the mRNA and protein levels, we investigated the change in the rates of DNA topoisomerase I protein synthesis *versus* degradation in human T lymphocytes following PHA stimulation. The increase in the mRNA parallels the increase in protein synthesis. However, the half-life of the enzyme protein was reduced to 9 h in proliferating T lymphocytes compared to a half-life of 36 h in resting lymphocytes. These results indicate that, in addition to the growth-regulated increase in the expression of DNA topoisomerase I, there was also a concomitant increase in the degradation of DNA topoisomerase I protein.

The topological state of DNA is an important determinant for DNA structure and function (1–5). Two different types of human DNA topoisomerases have been identified and characterized (6, 7). Type I DNA topoisomerase breaks and rejoins only one of the two DNA strands, while type II DNA topoisomerase breaks and rejoins both strands in each catalytic reaction. Through these two different types of reaction, the topological state of a DNA molecule is modulated, and the involvement of DNA topoisomerases in various DNA transactions, such as transcription, replication, recombination, and chromosomal segregation at mitosis, has either been documented or suggested (8–15). These important roles of DNA topoisomerases in modulating DNA functions prompted us to investigate the regulation of their expression during stimulated cell growth, when major changes in gene expression and replicative enzymes occur.

Previous studies from our laboratory have shown that the expression of DNA topoisomerase I is transiently elevated in human skin fibroblasts following phorbol ester stimulation (16). However, the regulation of the expression of DNA topoisomerase I during cell proliferation remains controversial (17–22). The DNA topoisomerase I protein level in proliferating cells has been shown to remain unchanged throughout different phases of the cell cycle (17–19). However, reduced DNA topoisomerase I activity in nonproliferating cells has been also reported (20–22). This apparent discrepancy may be explained by differences in the cell systems used in these studies (*e.g.* transformed *versus* untransformed cells). We therefore chose to study the regulation of DNA topoisomerase I gene expression in human T lymphocytes during cell growth because of the homogeneity of the human T lymphocyte population in the G_0 state of the cell cycle. Following PHA[1] stimulation, resting (G_0) T lymphocytes enter the cell cycle and undergo a programmed change synchronously. In this paper, we report our studies on the regulation of DNA topoisomerase I in human T lymphocytes stimulated with the mitogen PHA. We found that the rates of both protein synthesis and degradation of DNA topoisomerase I were increased upon the entry of T lymphocytes into proliferation.

EXPERIMENTAL PROCEDURES

Materials—PHA, a mitogen for human T lymphocytes, was purchased from Wellcome Diagnostics. All the other chemicals were of analytical grade and were purchased from Sigma. All tissue culture media were purchased from Life Biotechnologies, Inc. The GST gene fusion system was bought from P-L Biochemicals. Human DNA topoisomerase I cDNA was obtained as described previously (23). [^{35}S]Methionine and [α-^{32}P]dCTP were obtained from Amersham Corp. Reagents for gel electrophoresis were from Bio-Rad. Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and goat anti-rabbit IgG alkaline phosphatase conjugate used for immunoblotting analysis were purchased from Promega Biotec.

Cell Culture—Blood obtained from healthy donors was mixed with heparin sulfate (20 units/ml). Lymphocytes were isolated from the heparinized blood by Ficoll-Hypaque density gradient centrifugation and cultured at a density of 2×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum, penicillin/stretomycin (50 IU/ml and 50 μg/ml, respectively), and 1 mM glutamine in a controlled atmosphere of 5% CO_2 and 95% air (v/v) at 37 °C. For lymphocyte stimulation, PHA was added at a concentration of 0.1 mg/ml.

Expression and Purification of GST-TopI(DI) Fusion Protein—The 0.7-kilobase TopI(DI) cDNA fragment encoding amino acids 209–442 of human DNA topoisomerase I was subcloned into plasmid pGEX-KG, a GST gene fusion system (24). The strategy of subcloning TopI(DI) cDNA into plasmid pGEX-KG is shown in Fig. 1. The TopI(DI) cDNA, excised as an *Eco*RI insert, was cloned into the single *Eco*RI site on plasmid pGEX-KG. After insertion, the TopI(DI) cDNA was in frame with the GST gene. This expression plasmid, designated pGTopI-DI, expressed an M_r 50,000 protein upon isopropyl-1-thio-β-D-galactopyranoside induction in *Escherichia coli* BL21(DE3). To express the GST-TopI(DI) fusion protein in large quantities in *E. coli* BL21(DE3), 10 colonies of BL21(DE3) carrying pGTopI-DI were pooled and used to inoculate a 500-ml culture. When the cell density reached 3×10^7 cells/ml, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM, and incubation was continued for another 90 min. Cells were harvested by centrifugation and resuspended in 25 ml of PBST buffer (1

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[1] The abbreviations used are: PHA, phytohemagglutinin; GST, glutathione *S*-transferase; TopI, topoisomerase I.

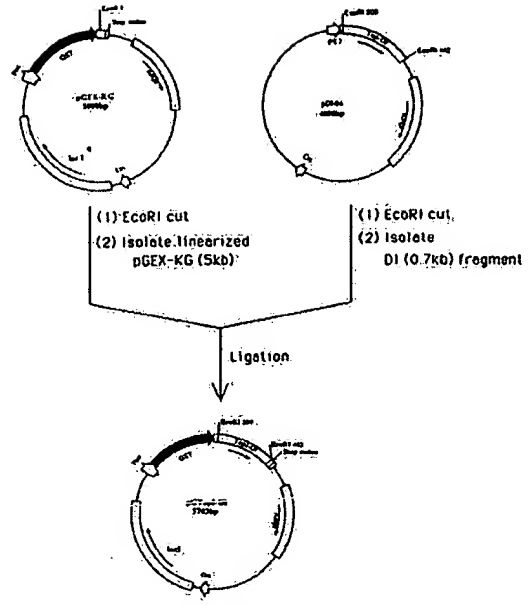

FIG. 1. **Construction of plasmid expressing GST-TopI(DI) fusion protein.** The 0.7-kilobase (*kb*) *Eco*RI fragment of human DNA topoisomerase I cDNA (clone DI), which encodes amino acids 209–442 of human DNA topoisomerase I, was inserted in the *Eco*RI site of plasmid pGEX-KG. The construction of pGTopI-DI from plasmids pGEX-KG and pDI-56 is diagrammatically shown.

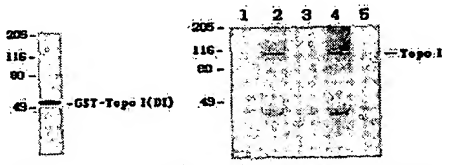

FIG. 2. **SDS-polyacrylamide gel electrophoresis of purified GST-TopI(DI) fusion protein (*left*) and characterization of rabbit anti-human DNA topoisomerase I antibodies (*right*).** *Left*, the GST-TopI(DI) fusion protein was expressed in *E. coli* BL21(DE3) and purified through a glutathione-agarose affinity column. Purified GST-TopI(DI) was examined on a 10% SDS-polyacrylamide gel. *Right*, GST-TopI(DI) was used as an antigen to raised anti-human DNA topoisomerase I antisera. The antisera were characterized by their ability to specifically immunoprecipitate human DNA topoisomerase I. *Lane 1*, immunoprecipitation with preimmune serum; *lanes 2* and *4*, immunoprecipitation with anti-GST-TopI(DI) antisera; *lane 3*, cells treated with camptothecin prior to immunoprecipitation with anti-GST-TopI(DI) antisera; *lane 5*, immunoprecipitation with anti-GST-TopI(DI) antisera in the presence of an excess amount of GST-TopI(DI).

× phosphate-buffered saline (140 mM NaCl, 2 mM KCl, 5 mM $Na_2H_2PO_4$, 2 mM NaH_2PO_4) plus 1% Triton X-100, pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by incubation with lysozyme (1 mg/ml) at 25 °C for 5 min. The lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was applied to a glutathione-agarose affinity column with unwanted proteins washed out by PBST buffer. The GST-TopI(DI) fusion protein was then eluted with 50 mM Tris-HCl containing 10 mM glutathione, pH 8.0. Chromatography on a glutathione-agarose affinity column resulted in purification of GST-TopI(DI) to >95% homogeneity (Fig. 2, *left*).

Preparation of Rabbit Polyclonal Antibodies against Human DNA Topoisomerase I—Antisera to human topoisomerase I were generated in female New Zealand White rabbits. Animals were injected subcutaneously with 200 μg of purified GST-TopI(DI) fusion protein mixed with Freund's complete adjuvant. The animals were boosted at 30-day intervals with antigen in Freund's incomplete adjuvant. Sera were collected at 7 and 14 days after boosting. The rabbit antisera were examined for their ability to immunoprecipitate human DNA topoisomerase I. As shown in Fig. 2 (*right*), PHA-stimulated human lymphocytes were labeled with [^{35}S]methionine for 2 h, and the labeled extracts were immunoprecipitated with the rabbit antiserum in the absence (*lane 4*) or presence (*lane 5*) of an excess amount of GST-TopI(DI). The results show an M_r 100,000 protein that is specifically reduced by an excess amount of GST-TopI(DI) and thus must represent DNA topoisomerase I. The identification of this M_r 100,000 protein as human DNA topoisomerase I was further confirmed by the specific reduction of the M_r 100,000 band intensity (>80% reduction) in cells treated with camptothecin (*lane 3*), a chemical causing covalent association of cellular DNA topoisomerase I with chromosomal DNA (30, 31). The bands of low molecular weight may represent breakdown products of DNA topoisomerase I since they were reduced by an excess amount of GST-TopI-(DI) during immunoprecipitation. This result indicates that GST-TopI-(DI) has been successfully used as an antigen to raise anti-human DNA topoisomerase I antiserum.

RNA Isolation and RNA Blotting—At various times post-stimulation with PHA, human lymphocytes were removed from cultures, and total cytoplasmic RNA was isolated according to the method described by Wilkinson (25). RNA was quantified by measurements of absorbance at 260 nm; fractionated on a 6% formaldehyde, 1% agarose gel (15 μg/well); and transferred to nitrocellulose (26). The resulting filter-bound RNA was prehybridized and then hybridized for 24 h with a nick-translated ^{32}P-labeled cDNA encoding human topoisomerase I (23). The specific radioactivity of this cDNA was ~10^9 dpm/μg of DNA. Following hybridization, the filters were washed and then exposed to Kodak XAR film with intensifying screens at −70 °C.

Immunoblotting and Immunoprecipitation—At various times after stimulation with PHA, the lymphocytes were harvested, lysed in lysis buffer (0.5% SDS, 1% β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5), and immediately boiled at 95 °C for 5 min. Protein concentrations were determined by the method of Bradford (28). Fifty μg of protein from each cell lysate was dissolved in Laemmli sample buffer (29) and boiled for 5 min prior to application to a 0.1% SDS, 7.5% acrylamide slab gel. For immunoblotting, electrophoresed samples were transferred from gel to Millipore polyvinylidene difluoride paper. The paper was then soaked in 3% gelatin for 2 h, followed by treatment with the antisera directed against human DNA topoisomerase I for 90 min in TBST buffer (125 mM NaCl, 0.05% Tween 20, and 25 mM Tris-HCl, pH 8.0). The paper was then washed with TBST buffer three times, followed by incubation with anti-rabbit antibody conjugated with alkaline phosphatase at room temperature for 30 min. After another three washes in TBST buffer, the paper was reacted with solution containing 0.33 mg/ml nitro blue tetrazolium, 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate for ~5 min to allow visualization of the band corresponding to DNA topoisomerase I. For immunoprecipitation, the lymphocytes (10^6 cells/ml) were labeled with [^{35}S]methionine (0.1 mCi/ml) in methionine-free medium and 10% dialyzed fetal bovine serum for various periods of time as indicated in the figure legends. At each indicated time point, cells were harvested and lysed in 50 μl of lysis buffer and immediately boiled at 95 °C for 5 min. Before immunoprecipitation, the cell lysates were pretreated with 20 μl of 20% (w/v) polyethylene glycol (M_r 8000) to remove DNA contaminant. The lysates were then diluted with 1 ml of radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Triton X-100, 150 mM NaCl, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride) and incubated with 3 μl of anti-topoisomerase I antisera at 4 °C for 1 h, followed by incubation with 20 μl of protein A-Sepharose CL-4B at 4 °C for 3 h. The immunoprecipitates were sequentially washed with 1 ml of buffer A (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.5% Nonidet P-40, and 0.02% SDS), 1 ml of buffer B (buffer A plus 1.0 M NaCl), and then 1 ml of buffer A. Immunoprecipitates were dissolved in 50 μl of Laemmli sample buffer (29), boiled for 5 min, and then analyzed by electrophoresis using a 10% SDS-polyacrylamide slab gel. To enhance the band intensity of DNA topoisomerase I, the gel was fluorographed with 2,5-diphenyloxazole.

RESULTS

Increased Accumulation of DNA Topoisomerase I mRNA in Human Lymphocytes following PHA Stimulation—Fig. 3*A* shows the results of [^{3}H]thymidine incorporation before and after PHA stimulation of human lymphocytes. Prior to PHA stimulation, with cells in G_0, DNA synthesis as measured by [^{3}H]thymidine incorporation was undetectable. DNA synthesis became detectable 24 h after PHA stimulation and was followed by a large increase 30–36 h after stimulation. These results suggest that 24 h after stimulation with PHA, cells were at the G_1/S boundary of the cell cycle, and they entered the S phase 30–36 h after PHA stimulation (Fig. 3*A*). Levels of the mRNA encoding DNA topoisomerase I following PHA stimulation are shown in Fig. 3*B*. Prior to PHA stimulation, the mRNA level

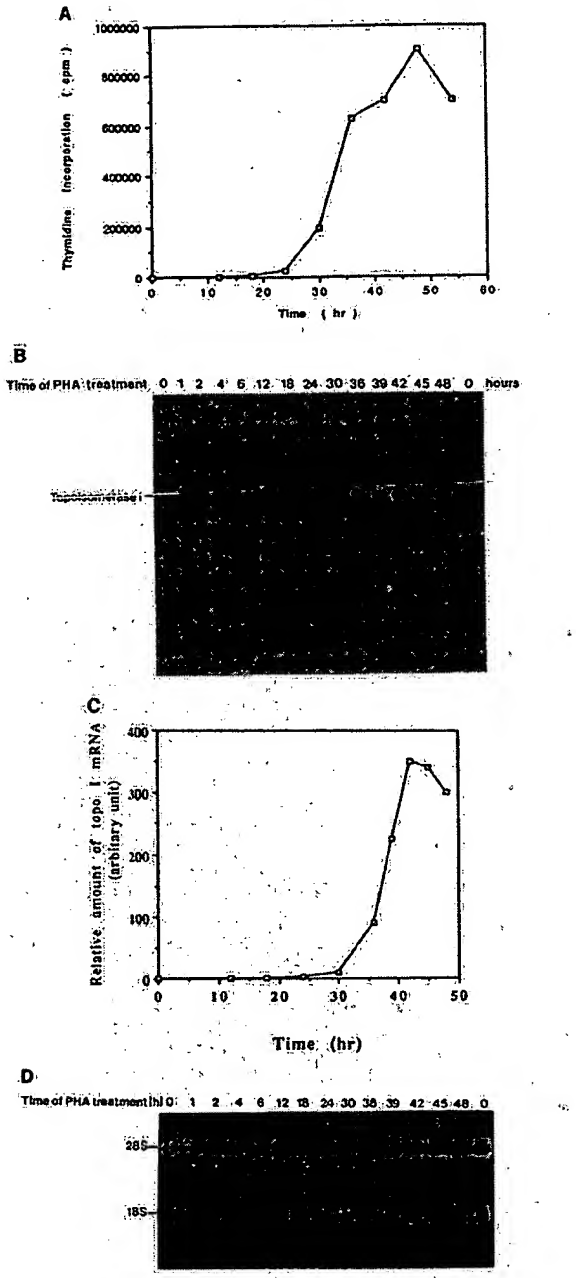

FIG. 3. **Time course of DNA topoisomerase I mRNA increase parallels entry of cells into proliferation in PHA-stimulated human lymphocytes.** *A*, graph showing the incorporation of [^{3}H]thymidine in human lymphocytes following PHA stimulation. *B*, human lymphocytes stimulated with PHA for the indicated times (0–48 h). Fifteen μg of total cytoplasmic mRNAs was then examined by Northern analysis using a labeled human topoisomerase I cDNA probe. *C*, quantitative analysis of the Northern results by densitometry. *D*, RNA gel stained with ethidium bromide. Equal intensities of both the 28 S and 18 S rRNA bands were observed in all samples.

was very low, which is observed only after long exposures (data not shown). An increase in this mRNA was observed after PHA stimulation; it became detectable 18–24 h after PHA stimulation after overexposing the autoradiograph (data not shown). A further increase occurred from 24 to 36 h post-stimulation as cells progressed through the S phase. The mRNA level peaked 42 h post-stimulation, after which it began to fall, although 48 h post-stimulation, it was still 75% of its maximum. Quantitation of the Northern results was performed using a densitometer, and the results are shown in Fig. 3*C*. The densitometric plot clearly showed that the level of DNA topoisomerase I mRNA increased >50-fold 42 h after stimulation with PHA. To rule out the possibility that the observed increase in mRNA could be due to inappropriate sample loading, the RNA gel was stained with ethidium bromide prior to transfer to a nitrocellulose membrane. As shown in Fig. 3*D*, ethidium bromide staining shows equal intensities of both the 28 S and 18 S rRNA bands for all RNA samples, indicating that an equal amount of RNA was loaded for every sample. One can thus conclude that DNA topoisomerase I gene expression is induced in human T lymphocytes upon their entry into proliferation.

Increased DNA Topoisomerase I Protein Level in Human T Lymphocytes following PHA Stimulation—Having demonstrated an increase in the level of mRNA of DNA topoisomerase I, we examined whether the increase in mRNA would result in an equivalent increase in the DNA topoisomerase I enzyme protein. Fig. 4*A* shows the results when the lymphocytes were harvested at various time points after PHA treatment and the level of protein measured by immunoblotting with anti-human DNA topoisomerase I antibody. The enzyme protein was detected prior to PHA stimulation and remained relatively constant during the first 12 h after PHA stimulation. An increase in topoisomerase I protein was first detected 18 h after PHA stimulation, but unlike the mRNA result, the increase in protein was only ~6-fold 42 h after stimulation with PHA. A duplicate SDS-polyacrylamide gel containing the same samples (50 μg/well) was made and stained with Coomassie Blue. The

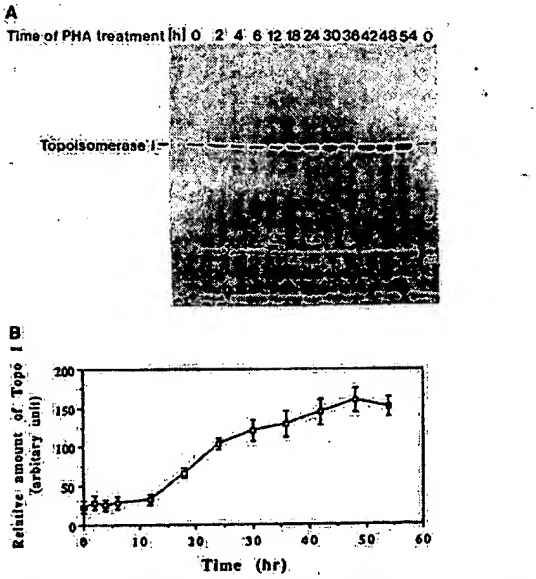

FIG. 4. **Increase in protein level of DNA topoisomerase I in PHA-stimulated human lymphocytes.** *A*, protein was harvested at the indicated time points after stimulation with PHA. Lymphocytes were washed and centrifuged, and the cell pellets were resuspended in 0.5% SDS, 1% β-mercaptoethanol, and 50 mM Tris, pH 7.5, and immediately boiled at 95 °C for 5 min. Samples (50 μg of protein/well) were electrophoresed and immunoblotted using antisera against human DNA topoisomerase I. *B*, shown is the statistical analysis of the increase in the protein level of DNA topoisomerase I in PHA-stimulated human lymphocytes. The data shown were derived from three individual experiments using a Molecular Dynamics computing densitometer. The three sets of data were collected and normalized as follows. The value of each time point (V_0 stands for value at time 0) obtained from densitometry was added to get a total value (T stands for total value, $T = V_0 + V_2 + V_4 + \cdots + V_{54}$). The value of each time point was then divided by the total value to obtain fractions at each time point (F_0 stands for fraction at time 0, $F_0 = V_0/T$). Fractions at the same time point from three individual experiments were analyzed in Stat View™ to obtain the mean ± S.D. values. The values of each time point are relative to the total value. The arbitary unit was obtained by mutiplying each fraction by 1000.

level of major proteins, such as actin, remained relatively constant in lymphocytes during the entire course of PHA stimulation (data not shown), indicating that the increase in DNA topoisomerase I in PHA-stimulated lymphocytes is specific. We have repeated this experiment three times, and similar results were obtained. Statistical analysis of the results is shown in Fig. 4*B*. An increase in topoisomerase I protein was first detected 18 h after PHA stimulation, and a 6-fold increase in DNA topoisomerase I protein was observed 42 h after stimulation with PHA. These results indicate that both the mRNA and the protein of DNA topoisomerase I are similarly regulated during the cell cycle. However, the quantitative differences in the increased levels between the protein and the mRNA remained unexplained.

Increased Synthesis and Degradation of DNA Topoisomerase I following PHA Treatment—The discrepancy between the increased amount of DNA topoisomerase I protein and the increased level of the mRNA suggested either that the mRNA is less efficiently translated during the proliferation of T lymphocytes or that an increased synthesis of protein is accompanied by an increased degradation of the protein. To evaluate the efficiency of translation of the mRNA, the amount of newly synthesized protein after PHA stimulation was measured by immunoprecipitation of [^{35}S]methionine-labeled proteins. Prior to PHA stimulation, newly synthesized topoisomerase I was undetectable. It became detectable 18–24 h after PHA stimulation and was followed by a large increase 36–48 h after stimulation (Fig. 5*A*). We have repeated this experiment three times, and similar results were obtained. Statistical analysis of the results is shown in Fig. 5*B*. Our results show that the increase in newly synthesized DNA topoisomerase I protein parallels the increase in the mRNA, thus excluding the possibility that the mRNA is less efficiently translated. However, as shown in Fig. 6, large differences in the half-lives of newly synthesized DNA topoisomerase I protein between resting and PHA-stimulated lymphocytes were observed. Since very little new DNA topoisomerase I protein was synthesized in the resting lymphocytes, labeling with [^{35}S]methionine was carried out for 24 h. In contrast, PHA-stimulated lymphocytes synthesize large quantities of new DNA topoisomerase I protein and were thus labeled with [^{35}S]methionine for only 2 h. The decay of labeled DNA topoisomerase I protein in the PHA-stimulated lymphocytes (Fig. 6*B*) is more rapid than in resting lymphocytes (Fig. 6*A*). Analysis of the decay on a logarithmic scale gave half-lives of the enzyme protein in resting and PHA-stimulated lymphocytes of 36 and 9 h, respectively (Fig. 6*C*). Thus, the shorter half-life of DNA topoisomerase I protein in the proliferating T lymphocytes can account for the smaller increase in protein when compared with the mRNA. These experiments also indicate that, in addition to the growth-regulated expression of DNA topoisomerase I, there is also a growth-dependent degradation of the protein.

During the study of the half-lives of DNA topoisomerase I in resting and proliferating T lymphocytes, we observed some higher molecular weight proteins that could also be immunoprecipitated by anti-topoisomerase I antibody in PHA-stimulated lymphocytes (Fig. 6*B*), but not in resting lymphocytes (Fig. 6*A*). These higher molecular weight proteins may result from various modifications of DNA topoisomerase I. However, at this time, we also cannot rule out the possibility that these higher molecular weight proteins may represent immunoprecipitation artifacts.

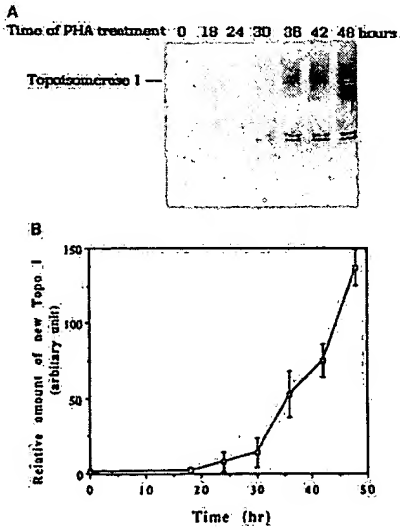

FIG. 5. **Increase in new DNA topoisomerase I protein synthesis upon entry of human T lymphocytes into proliferation.** *A*, new DNA topoisomerase I protein synthesis was measured in PHA-stimulated lymphocytes by [^{35}S]methionine pulse labeling for 2 h, followed by immunoprecipitation with anti-human topoisomerase I antibody; *B*, statistical analysis of the increase in new DNA topoisomerase I protein synthesis in PHA-stimulated human lymphocytes. The data were derived from three individual experiments and were analyzed as described in the legend of Fig. 4*B*.

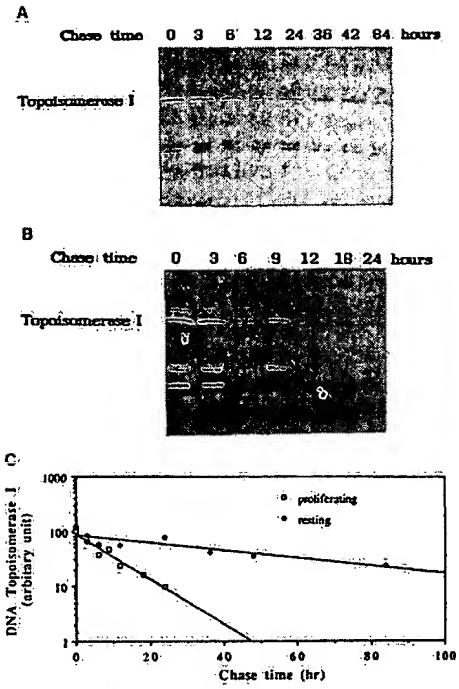

FIG. 6. **Increase in human DNA topoisomerase I degradation upon entry of human T lymphocytes into proliferation.** Resting and PHA-stimulated human lymphocytes were pulse-labeled with [^{35}S]methionine, followed by an unlabeled methionine chase. At various time points of chase, cells were harvested, and the amount of labeled DNA topoisomerase I protein was compared after immunoprecipitation. The decay of labeled DNA topoisomerase I protein in resting and PHA-stimulated lymphocytes is shown in *A* and *B*, respectively. Quantitative analysis of the decay of labeled DNA topoisomerase I protein on a logarithmic scale is shown in *C*.

DISCUSSION

In this study, we have examined the changes in DNA topoisomerase I mRNA and protein synthesis following PHA stimulation of resting lymphocytes. Treatment with PHA resulted in cell proliferation accompanied by an increase in mRNA levels. Although an increased level of protein was also noted, this increase was found to be quantitatively much less as a result of

increased protein degradation. These results may help to clarify the current controversy on the regulation of DNA topoisomerase I during cell growth. The demonstration that DNA topoisomerase I is regulated at both transcriptional and translational levels during growth stimulation suggests that previous reports showing that DNA topoisomerase I protein levels are similar in different phases of the cell cycle may be incorrect (17–19). In this study, DNA topoisomerase I protein increased 6-fold with the onset of lymphocyte proliferation, whereas the mRNA increased >50-fold, providing strong evidence that the expression of this enzyme is regulated by growth stimulation. Using guinea pig lymphocytes for a similar study, Taudou *et al.* (32) found a 4–5-fold increase in DNA topoisomerase I activity after 72 h of concanavalin A stimulation. Similar results were also observed in various other cell lines, although the increase during growth stimulation in human T lymphocytes was greater than in any other tested cell lines, including human skin fibroblasts, HeLa cells, etc. (data not shown).

Studies on [^{3}H]thymidine incorporation in PHA-stimulated lymphocytes demonstrated a long latent phase prior to cell proliferation. Incorporation was detectable at 24, with a more dramatic increase occurring 30–36 h post-stimulation, suggesting that DNA replication was initiated ~24 h post-stimulation in the majority of T lymphocytes. The time course of the increase in the mRNA of DNA topoisomerase I appears to parallel that of thymidine incorporation. It was detectable at 18–24 h, and a more dramatic increase occurred 30–48 h post-stimulation. These results suggest that DNA topoisomerase I is initially expressed just before the G_1/S boundary and accumulates rapidly during the S phase of the cell cycle. Since previous studies have demonstrated that maximum accumulation of topoisomerase II protein and mRNA occurs several hours after the peak of thymidine incorporation (33), our observations may imply that DNA topoisomerase I participates in the initiation/elongation phase of DNA replication, while DNA topoisomerase II is involved in the termination stage of DNA replication, during which multiply interlocked daughter DNA molecules are to be segregated (8, 10).

DNA topoisomerase I protein increased 6-fold following PHA stimulation, which was significantly less than the increase in the level of topoisomerase I mRNA (>50-fold). Our results suggest that the increase in new DNA topoisomerase I protein is simultaneously accompanied by an increased degradation of the protein. This observation raises the question of whether the cells really need DNA topoisomerase I to enter proliferation. Although we cannot answer this question at this time, the entry of cells into proliferation is accompanied by an increase in both DNA synthesis and RNA transcription, and one would predict a need for increased DNA topoisomerase I activity to relax the topological strain of DNA generated during DNA replication and transcription. However, since DNA topoisomerase I lacks sequence specificity in its relaxation reaction, one could postulate that DNA topoisomerase I may be potentially dangerous to genomic stability if not degraded after relaxing the DNA strain generated during DNA replication and transcription. The increase in both the synthesis and degradation of the enzyme thus provides a mechanism for the cell to meet the need of DNA topoisomerase I upon entry into proliferation, but prevents potential danger from overexpression of this enzyme. This viewpoint is further supported by the observation that other cell cycle-regulated gene products, such as cdc2 and proliferating cell nuclear antigen (34, 35), are also rapidly synthesized and degraded in proliferating cells.

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Helicobacter pylori Increases Proteasome-mediated Degradation of $p27^{kip1}$ in Gastric Epithelial Cells[1]

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ABSTRACT

***Helicobacter pylori* infection is associated with increased gastric epithelial cell turnover and is a risk factor for noncardia gastric cancer. *H. pylori* reduces the expression of p27 protein, a cyclin-dependent kinase inhibitor of the G_1 to S-phase cell cycle transition and gastric tumor suppressor gene. Although cell cycle dysregulation associated with decreased p27 may contribute to gastric carcinogenesis, how *H. pylori* reduces p27 in gastric epithelial cells remains unknown. In the present study, we investigated the mechanisms of the p27 decrease, using AGS and MKN28 gastric epithelial cells cocultured with *H. pylori* strains under conditions of defined cell cycle distribution. The expression of p27 protein was reduced by *H. pylori* in a dose- and time-dependent manner. Northern blot and pulse-chase analyses revealed that this reduction was not regulated at a transcriptional level but by accelerated p27 degradation via a proteasome-dependent pathway. Despite up-regulation of the proteasome-dependent degradation of p27 protein, neither threonine 187-phosphorylated p27 nor skp2 (the ubiquitin ligase for p27) were increased. Furthermore, *H. pylori* impaired p27 ubiquitination and did not increase global proteasomal function. These results indicate that *H. pylori* increases the degradation of p27 through a proteasomal pathway distinct from the physiological pathway that degrades p27 during cell cycle progression. Putative virulence genes of *H. pylori* (*cagA*, *cagE*, or *vacA*) played no role in reducing p27 expression. Increased degradation of p27 by *H. pylori* through a proteasome-dependent, ubiquitin-independent pathway may contribute to the increased risk of gastric cancer associated with chronic *H. pylori* infection.**

INTRODUCTION

Epidemiological studies have demonstrated that gastric infection with the Gram-negative bacterium *Helicobacter pylori* is associated with an increased risk of developing noncardia gastric cancer (1–3). Furthermore, eradication of *H. pylori* may prevent gastric cancer in high-risk populations with chronic gastritis (4) and can reduce the rate of progression from intermediate preneoplastic gastric lesions of intestinal metaplasia and atrophy (5). More direct evidence implicating *H. pylori* as a gastric carcinogen has come from the demonstration of gastric cancer in the Mongolian gerbil following experimental infection by *H. pylori* (6, 7) and from C57/BL6 mice infected with the related gastric bacterium *H. felis* (8).

How *H. pylori* promotes gastric carcinogenesis is not known. However, studies in humans and in animal models have demonstrated that *H. pylori* increases the percentage of gastric mucosal epithelial cells displaying markers of proliferation and apoptosis (9, 10). Because increased cell turnover is a common precursor of neoplastic transformation (11), investigating the link between *H. pylori* and altered gastric epithelial cell cycling may therefore provide important information regarding the mechanisms of gastric carcinogenesis associated with chronic *H. pylori* infection.

Progression of cells through the cell cycle is controlled by interactions between cell cycle control proteins (cyclins) and their catalytically active CDKs.[3] The activity of each cyclin-CDK complex is in turn regulated by several different mechanisms, the most important being negative regulation by CDK inhibitors (12). Mutation or aberrant expression of specific cell cycle-regulatory proteins is common in tumors, suggesting that these proteins are critical targets during carcinogenesis (13). $p27^{kip1}$ (p27) is a CDK inhibitor, whose major targets are the cyclin E-CDK2 and cyclin D-CDK4/6 complexes, that governs cell cycle transition from late G_1 to S phase (14). The amount of p27 is mainly regulated by posttranslational ubiquitin-proteasome-mediated proteolysis. The cell cycle-dependent degradation of p27 is dependent on phosphorylation at Thr^{187} in late G_1 phase by CDK2, under positive regulation by cyclin E. Thr^{187} phosphorylation is a necessary prerequisite for the sequential addition of ubiquitin molecules by a ubiquitin ligase complex containing the F-box protein skp2 (for review, see Ref. 15). Polyubiquitination of p27 then targets p27 for degradation in the proteasome, thus removing the p27 cell cycle "brake," allowing cells to transition from G_1 to S phase. Loss of p27 function therefore accelerates cell cycle progression and predisposes cells to malignant transformation, as is well illustrated by the observation of increased tumor incidence in hemizygous and homozygous p27-deleted mutant mice after carcinogen exposure (16). For this reason, p27 has been termed a haploinsufficient tumor suppressor gene (16), and decreased expression of p27, probably due to increased proteasomal degradation of p27 after ubiquitination (17, 18), has been demonstrated to be associated with a poor prognosis in several types of cancer, including gastric cancer (14, 19, 20).

Recent studies by our group and others have shown that the level of p27 protein in gastric epithelial cells is decreased by *H. pylori*, both in chronic gastritis (21) and in intestinal metaplasia (22). Furthermore, p27 expression is reduced in gastric epithelial cells exposed to *H. pylori* acutely (23) and chronically (21), suggesting that the bacterium itself rather than the associated inflammatory response present in the gastric mucosa can rapidly and permanently down-regulate epithelial cell p27. Because decreased p27 may be an important step linking *H. pylori* to hyperproliferation and gastric carcinogenesis, we investigated the cellular and molecular mechanisms responsible for this effect of *H. pylori*.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. AGS human gastric epithelial cells (CRL-1739; American Type Culture Collection, Manassas, VA) and MKN28 human gastric epithelial cells (JCRB0253; Japan Health Sciences Foundation) were maintained in an atmosphere of 5% CO_2 at 37°C in Ham's F-12 medium (AGS) or RPMI 1640 (MKN28) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) without antibiotics in 75-cm^2 tissue culture flasks (BD Biosciences, San Jose, CA). For synchronization of the cell cycle at G_0-G_1 phase, cells were serum deprived for 48 h.

***H. pylori* Strains and Culture Conditions.** Coculture experiments were performed, as described previously (23), using wild-type *H. pylori* strain 60190 (ATCC49503), a *cagA*-positive and *vacA*-positive strain isolated from a patient

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[3] The abbreviations used are: CDK, cyclin-dependent kinase; AMC, 7-amino-4-methylcoumarin.

with nonulcer dyspepsia, its isogenic *cagA*-negative mutant, its isogenic *vacA*-negative mutant, or its isogenic *cagE* (=*picB*)-negative mutant [kindly provided by M. Blaser (New York University); Refs. 24–26]. Five minimally passaged clinical strains [GC102 and A855 (provided by Mark Kidd; Yale University, New Haven, CT) and J68, B107, and B166 (provided by Richard Peek; Vanderbilt University, Nashville, TN] were also used (27, 28). GC102 was isolated from a 58-year-old man with a diffuse-type antral adenocarcinoma, A855 was isolated from a 37-year-old woman with chronic atrophic pangastritis, J68 was isolated from a 33-year-old woman who had a duodenal ulcer, B107 was isolated from a 60-year-old woman with gastric and duodenal erythema, and B166 was isolated from a 61-year-old woman with duodenal erythema. Bacteria were maintained on trypticase soy agar containing 5% sheep blood (BD Biosciences) incubated at 37°C in 5% CO_2 for a minimum of two and a maximum of four passages from frozen stocks. Inocula for coculture were diluted from suspensions that had been prepared from 48-h subcultures and adjusted by comparison of absorbance to McFarland standards. *H. pylori* bacteria were added to AGS cells at a ratio of 1:200 in all experiments, unless otherwise stated in the figure legends. To verify the viability of *H. pylori* after incubation in culture media, the media were serially diluted 10-fold, 100 μl of each dilution were plated on agar plates that allow optimal growth of *H. pylori*, and colony counts were determined 48 h after plating.

Cell Cycle Analysis. Adherent and floating epithelial cells were collected together and fixed in 70% ethanol. Cell pellets were suspended in 400 μl of 0.2 mg/ml propidium iodide containing 0.6% NP40 (ICN Pharmaceuticals, Costa Mesa, CA) plus the same volume of 2 mg/ml RNase (Sigma Chemical Co., St. Louis, MO) and then incubated in the dark at room temperature for 30 min. Data acquisition and analysis were performed on a FACSort instrument equipped with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cell cycle analysis was performed with ModFIT software (Becton Dickinson Immunocytometry Systems). All experiments were performed at least three times and gave similar results.

Protein Extraction and Western Blotting. Cells were collected with a rubber policeman, washed twice in ice-cold PBS, and resuspended in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1.0 mM EDTA, 1.0 mM DTT, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1.0 mM sodium fluoride, and 0.1 mM sodium orthovanadate (adjusted to pH 7.5), plus the protease inhibitors leupeptin (10 mg/ml), aprotinin (10 mg/ml), and 1.0 mM phenylmethylsulfonyl fluoride. The suspended cells were sonicated on ice (twice for 15 s) with a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) and then centrifuged at 13,000 × *g* for 10 min at 4°C to yield soluble cell lysates. Protein concentrations were determined using the BCA Protein Assay (Pierce Chemical Co., Rockford, IL). For Western blotting, 30 μg of a total cell lysate were subjected to SDS-PAGE. The proteins were then transferred to Immobilin-P membranes (Millipore, Bedford, MA). The primary antibodies used were mouse monoclonal antibodies to $p27^{kip1}$ (clone 57; BD Biosciences), cyclin A (Upstate Biotechnology, Lake Placid, NY), cyclin D1/2 (Upstate Biotechnology), ubiquitin (clone P4D1; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and β-actin (Sigma Chemical Co.) and rabbit polyclonal antibodies to Thr^{187}-phosphorylated p27 (PT-187; Zymed Laboratories Inc., San Francisco, CA), $p45^{skp2}$ (clone SKP2–2B12; Zymed Laboratories Inc.), cyclin E [kindly provided by J. Singer (Brown University)], CDK2 (Upstate Biotechnology), and $p21^{cip1}$ (sc-397, Santa Cruz Biotechnology Inc.). Immune detection was performed using the enhanced chemiluminescence Western blotting detection system (Perkin-Elmer Life Sciences Inc., Boston, MA). β-Actin immunoblotting was performed to verify that equal amounts of protein had been loaded in each lane. Quantitative densitometric analysis was performed using NIH Image software.

Northern Blotting. Total RNA was prepared with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Ten μg of total RNA were electrophoresed on a 1% agarose denaturing gel, transferred to Hybond-N membrane (Amersham Biosciences Corp., Piscataway, NJ) by capillary electrophoresis, and fixed with UV light. The membrane was hybridized with a ^{32}P-labeled human p27 cDNA probe (29). After washing, the membrane was exposed to film with intensifying screens at −80°C.

Pulse-chase Analysis of p27 Degradation. Cells were serum starved for 48 h and metabolically radiolabeled with [^{35}S]methionine and [^{35}S]cysteine (200 μM; Perkin-Elmer Life Sciences Inc.) for 2 h. The culture medium was then replaced by serum-free, methionine-containing medium. After 2, 4, and 6 h of coculture with *H pylori*, cells were harvested, and lysates were immunoprecipitated with anti-p27 antibody. Immunoprecipitates were subjected to SDS-PAGE, and signals were surveyed by autoradiography.

***In Vitro* p27 Ubiquitination Assay.** [^{35}S]Methionine-labeled p27 was prepared with the coupled *in vitro* transcription/translation system (Promega, Madison, WI) using plasmid pcDNA3/p27 (30). Cells were serum starved for 48 h and then cocultured with or without *H. pylori* for 6 h. Extracts were prepared as described by Nguyen *et al.* (31), with minor modifications. Briefly, plates were washed twice with ice-cold PBS and once with cold hypotonic buffer [20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 5 mM KCl, and 1 mM DTT], resuspended in hypotonic buffer, left to stand on ice for 30 min, and then centrifuged at 13,000 × *g* for 30 min at 4°C, and supernatants were collected. Ten μl of radiolabeled p27 were then incubated with 200 μg of cell extracts supplemented with 1 mg/ml methylated ubiquitin (Boston Biochem, Cambridge, MA), 2 μM ubiquitin aldehyde (Boston Biochem), 200 μM MG-132 (Calbiochem, San Diego, CA), and an ATP regeneration system (25 mM phosphocreatine, 10 μg/ml creatine kinase, and 1 mM ATP) at 30°C in a total volume of 50 μl for the times indicated in the figure legends. The reactions were stopped by the addition of SDS sample buffer, and the products were resolved by SDS-PAGE. Ubiquitinated p27 forms were identified by fluorography.

***In Vitro* Proteasome Activity Assay.** Cells were collected and resuspended in a lysis buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT. They were then sonicated twice for 15 s and centrifuged at 13,000 × *g* for 10 min, and the supernatants were collected as whole cell extracts. Twenty-μg aliquots of cell extracts were then incubated in reaction buffer [25 mM HEPES, 0.5 mM EDTA (pH 7.6)] in quadruplicate for 30 min at 37°C with the following fluorogenic substrates: (*a*) chymotrypsin-like activity, Suc-Leu-Leu-Val-Tyr-AMC; (*b*) peptidylglutamyl peptide hydrolyzing activity, Z-Leu-Leu-Glu-AMC; and (*c*) trypsin-like activity, Z-Ala-Arg-Arg-AMC, 2HCl (all from Calbiochem). The amount of product (free AMC) was measured by Fluorocounter (Packard Bio Science Co., Meriden, CT) with an excitation filter of 360 nm and an emission filter of 460 nm.

***In Vitro* Assay for CDK2-associated Activity.** The *in vitro* CDK2-associated kinase assay was performed as described previously (32). Cells were collected, sonicated twice for 15 s in lysis buffer, and centrifuged, and the supernatant fraction was collected. Immunoprecipitation with 1 μg of anti-CDK2 polyclonal antibody was performed using protein A-Sepharose beads (Sigma Chemical Co.), followed by washing of the beads four times with lysis buffer and twice with reaction buffer [50 mM HEPES, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate (pH 7.5)]. The final pellets were resuspended in 45 μl of reaction buffer containing 2 mg of histone H1 (Calbiochem) and 5 mCi of [γ-^{32}P]ATP and incubated for 30 min at 30°C. The reaction mixture was then subjected to SDS-PAGE, and the intensity of phosphorylation of the histone H1 substrate was determined by autoradiography.

RESULTS

Cell Cycle-dependent Expression of p27 Protein in AGS Cells. The expression of p27 protein was low in AGS cells during the exponential phase of growth in medium containing 10% serum (Fig. 1*A*). Under these conditions, expression of skp2, the ubiquitin ligase for p27, was abundant. In comparison, after 48 h of serum deprivation, AGS cells accumulated in the G_0-G_1 phase of the cell cycle (Fig. 1*B*) in association with high expression of p27 and low expression of phosphorylated p27 and skp2, consistent with reduced degradation of p27 protein in the G_0-G_1 phase of the cell cycle. Cyclin E, the major positive regulator of the G_1 to S-phase transition was also at a low level in these cells. After treatment of AGS cells with the proteasome inhibitor MG-132, both p27 and phosphorylated p27 expression were markedly increased, consistent with a decrease in proteasomally mediated degradation of p27.

***H. pylori* Reduces p27 Protein Expression Dose-dependently, Irrespective of Cell Cycle Effects.** The level of p27 protein changes considerably during normal cell cycle progression. To examine the effects of *H. pylori* on p27, it was therefore necessary to separate

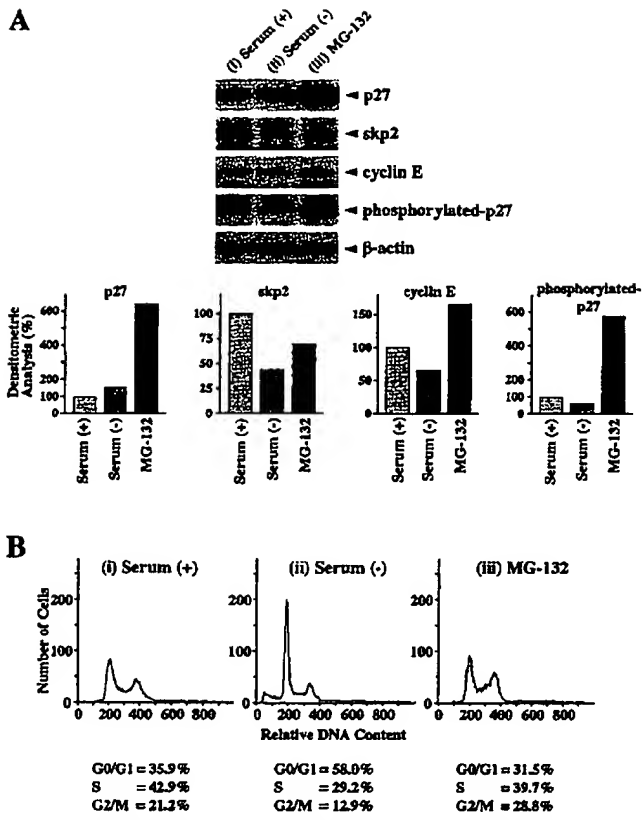

Fig. 1. Correlation of p27-related protein expression with specific cell cycle distributions in AGS cells. AGS cells were cultured (*a*) in normal medium [*Serum* (+)], (*b*) for 48 h in serum-free medium [*Serum* (−)], and (*c*) in the presence of the proteasome inhibitor MG-132 (10 μM). The *bottom graphs* in *A* show the densitometric analysis of protein expression under these conditions. *A*, protein analysis by Western blot, with β-actin as control. *B*, flow cytometric analysis of cell cycle phase distribution, with percentages of cells in each phase as shown.

changes in p27 levels due to altered cell cycle progression induced by *H. pylori* from changes in p27 due to effects of *H. pylori* that are not related to *H. pylori*'s effects on the cell cycle. To do this, we deprived AGS cells of serum for 48 h to inhibit the G_1 to S-phase cell cycle transition and then added *H. pylori* in the absence of serum. Under these conditions, the addition of *H. pylori* did not alter cell cycle phase distribution significantly (Fig. 2*A*), yet there was a dose-dependent decrease of p27 protein (Fig. 2*B*, *Lanes 1–5*). Thus, the reduction of p27 induced by *H. pylori* was not due to the physiological down-regulation of p27 during cell cycle progression. The addition of *H. pylori* to serum-fed, exponentially growing AGS cells also resulted in a dose-dependent decrease in p27 expression (Fig. 2*B*, *Lanes 6–9*), but the effect was less marked in these proliferating cells.

Reduction of p27 Protein Is due to Its Increased Degradation. The addition of *H. pylori* did not alter the abundance of steady-state p27 mRNA as determined by Northern blotting (Fig. 3*A*). Because the regulation of p27 protein abundance under physiological (cell cycle-related) conditions is known to be predominantly posttranslational, we therefore examined the effect of *H. pylori* on the degradation of p27 protein. Metabolically radiolabeled p27 was more rapidly degraded in the presence of *H. pylori* (Fig. 3*B*), indicating that the down-regulation of p27 by *H. pylori* is due to increased p27 protein degradation.

Effect of *H. pylori* on Expression of p27 Protein Degradation Intermediates and Its Ubiquitin Ligase, skp2. The degradation of p27 during the cell cycle is known to be largely through the ubiquitin-proteasome pathway. The addition of the proteasome inhibitor MG-132 at a concentration of 20 μM that did not influence *H. pylori* viability (as assessed by colony counts 48 h after plating the media; data not shown) abolished the decrease of p27 induced by *H. pylori* (Fig. 4*A*). This result indicates that the decrease in p27 induced by *H. pylori* depends on proteasomal activity. However, the reduction in levels of p27 protein caused by *H. pylori* was accompanied by decreased expression of phosphorylated p27 and skp2 (Fig. 4*B*), thus the decrease in p27 induced by *H. pylori* was proteasome dependent, but not through the physiological phosphorylation-ubiquitination pathway associated with increased skp2 expression.

Although Shirane *et al.* (33) reported that under some circumstances p27 may be degraded into a M_r 22,000 proteolytic product through a ubiquitin proteasome-independent pathway, the M_r 22,000 proteolytic product of p27 was not observed in any of these experiments (data not shown).

***H. pylori* Paradoxically Decreases CDK2-associated Kinase Activity.** Phosphorylation of p27 at Thr^{187} in the physiological ubiquitin-proteasome pathway is mediated by cyclin E-associated CDK2 activity, and a reciprocal relationship exists between cyclin E-associated CDK2 activity and the expression of p27. However, the reduction of p27 and phosphorylated p27 that we observed after the addition of *H. pylori* was associated with a *decrease* in CDK2 kinase activity (Fig. 5*A*). This was not due to an inhibition of the binding of CDK2 by p27 because the expression of p27 was reduced to a similar extent

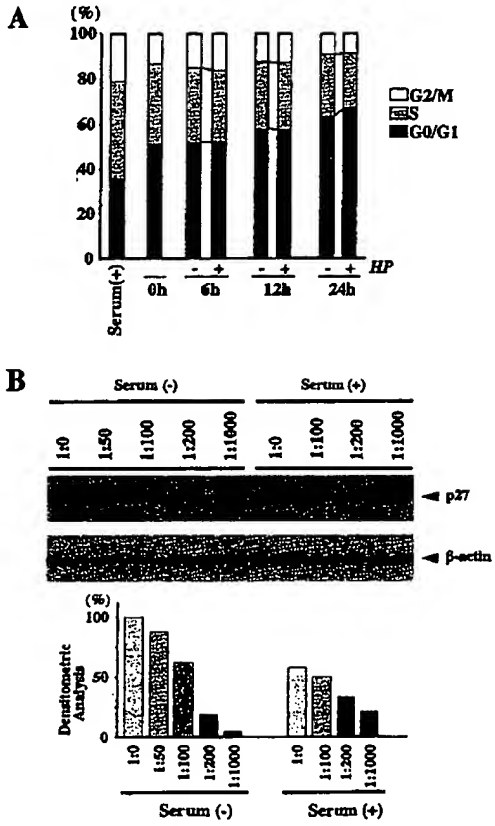

Fig. 2. *A*, cell cycle phase distribution of AGS cells in the presence or absence of *H. pylori*. AGS cells were synchronized by 48 h of serum starvation (0 h) and harvested for flow cytometry 0, 6, 12, and 24 h after the addition of *H. pylori* (+) or medium alone (−). The first column illustrates the results for AGS cells growing exponentially in 10% serum. [*Serum* (+)]. *B*, alteration of p27 protein expression by *H. pylori*. AGS cells were starved of serum for 48 h to induce synchronization (*Lanes 1–5*) or grown continually in medium containing serum (*Lanes 6–9*), and *H. pylori* was added at the indicated ratios. The expression of p27 protein was measured by immunoblot after 12 h of coculture. The *bottom graph* shows the densitometric analysis of the expression of p27. The decrease of p27 was dependent on the concentration of *H. pylori* and was more marked in the serum-starved cells.

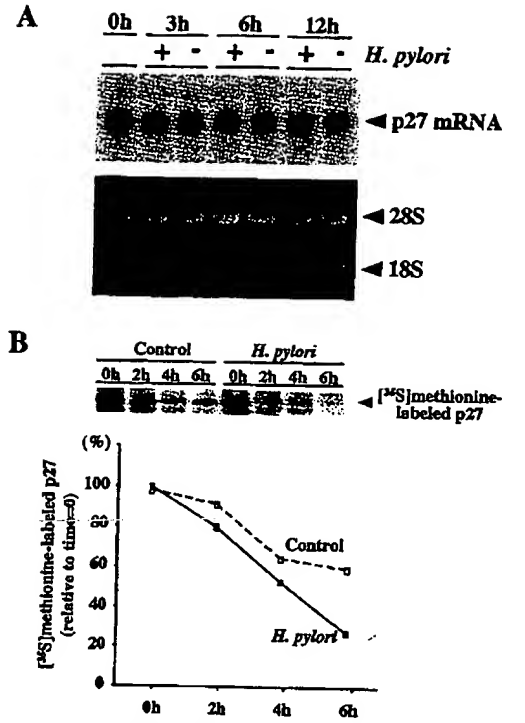

Fig. 3. *A*, Northern blot analysis of p27 mRNA in AGS cells after 0, 3, 6, and 12 h of coculture with *H. pylori*, demonstrating that the abundance of p27 mRNA was not altered by *H. pylori*. *B*, pulse-chase analysis of the turnover rate of p27. The *bottom graph* shows the densitometric analysis of the radiolabeled p27, relative to time 0 (100%). The addition of *H. pylori* increases the rate of degradation of ^{35}S-labeled p27.

by *H. pylori* in both the CDK2 immunoprecipitate and the supernatant (Fig. 5*B*). To determine the mechanism of the decrease of CDK2 activity associated with decreased p27, we examined the expression of other regulatory partners of CDK2 activity and of other proteins involved in the transition from the G_1 to S phase of the cell cycle (Fig. 5*C*). Both cyclin E and cyclin A were also decreased by *H. pylori*. In contrast to these reductions in cyclin E, cyclin A, and p27, *H. pylori* did not alter cyclin D1/2 or the CDK inhibitor p21.

***H. pylori* Inhibits Ubiquitination of p27.** During the cell cycle-dependent regulation of p27, the degradation of p27 via the ubiquitin-proteasome pathway is dependent on p27 ubiquitination mediated by skp2. Because a skp2-independent ubiquitination pathway has also been reported (34), the decrease of skp2 expression by *H. pylori* that we observed does not preclude *H. pylori* from increasing the ubiquitination of p27. However, the *in vitro* p27 ubiquitination assay demonstrated that *H. pylori* decreased the ability of AGS cells to ubiquitinate p27 (evident from comparing *Lanes 5* and *6* in Fig. 6*A* with *Lanes 2* and *3*, respectively). As a control in this assay, p27 ubiquitination activity was greater in lysates from exponentially growing AGS cells (*Lane 7*) compared with serum-starved cells (*Lane 3*), consistent with increased ubiquitin-dependent p27 degradation during S phase. Additionally, the expression of several ubiquitinated proteins that may normally be detected by anti-ubiquitin antiserum when proteasomal function is inhibited were decreased by *H. pylori* (Fig. 6*B*). Taken together, these results indicate that *H. pylori* does not increase but instead decreases the ubiquitination of p27 and possibly other proteins.

Effects of *H. pylori* on Proteasomal Activity. Our results show that the proteasome-dependent degradation of p27 by *H. pylori* is associated by neither up-regulation of phosphorylation nor increased ubiquitination of p27. However, it is conceivable that our findings could be explained by *H. pylori* markedly accelerating p27 degradation at the level of increased proteasomal function, despite low concentrations of the intermediary forms of p27 (Thr^{187}-phosphorylated p27 and ubiquitinated p27). We therefore measured the *in vitro* proteasomal activity of lysates from AGS cells that were cultured with *H. pylori* to test the hypothesis that *H. pylori* increases proteasomal activity nonspecifically. However, after the addition of *H. pylori*, the three major proteasome activities examined were decreased (Fig. 7), as determined by proteolytic cleavage of representative proteasomal substrates. These results indicate that the increased degradation of p27 by *H. pylori* is not due to an increase in global proteasomal function, but rather due to a ubiquitin-independent, proteasome-dependent pathway.

Effects of *H. pylori* on MKN28 Cells. We have previously reported that *H. pylori* inhibits the G_1 to S-phase cell cycle progression through a p53-independent mechanism (23). Because AGS cells possess wild-type p53 (35), to determine whether any of the changes we observed in AGS cells were dependent on functional p53, we also examined the effect of *H. pylori* on p27 and related molecules in MKN28 gastric epithelial cells that have a missense mutation in p53 (codon 251, isoleucine to leucine; Ref. 36). As seen in Fig. 8, MKN28 cells showed reductions in p27, cyclin E, and cyclin A that were similar to those observed in AGS cells (Fig. 5*C*), indicating that these changes are independent of p53 function.

Effect of *H. pylori*-related Factors on p27 Expression. The reduction of p27 induced by *H. pylori* was dependent on direct contact between live *H. pylori* and AGS cells because it could be abolished by separating AGS cells from *H. pylori* by a Transwell membrane (Nalge

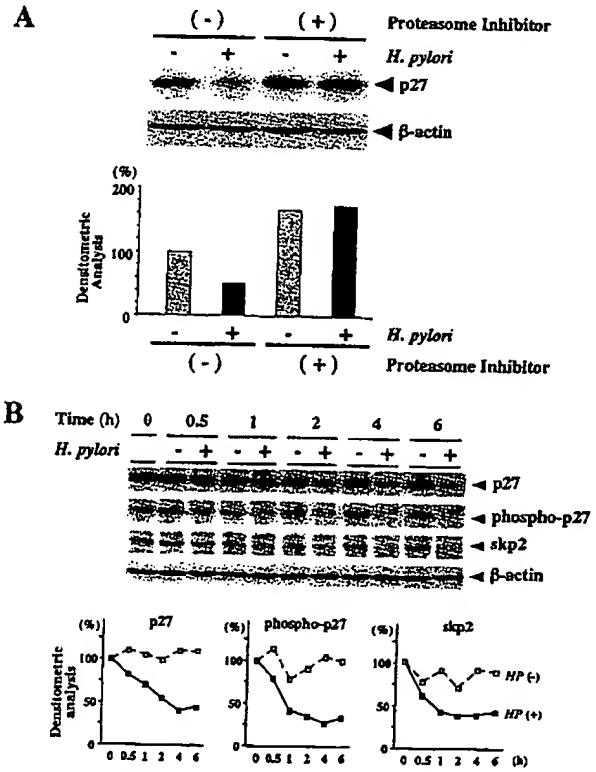

Fig. 4. *A*, p27 degradation by *H. pylori* is proteasome dependent. AGS cells and *H. pylori* were cocultured for 6 h in the absence (−) or presence (+) of the proteasome inhibitor MG-132 (20 μM). Western blot demonstrates that the decrease in p27 expression induced by *H. pylori* is abolished by MG-132. The *bottom graph* shows the densitometric analysis of p27 expression in this blot, normalized for β-actin. *B*, effects of *H. pylori* on total p27, Thr^{187}-phosphorylated p27, and skp2 ubiquitin ligase. AGS cells were serum deprived for 48 h, and then *H. pylori* (+) or serum-free medium only (−) was added, and cells were harvested at the times indicated. The *bottom graph* shows the densitometric analysis of specific protein expression by immunoblot after normalization for β-actin.

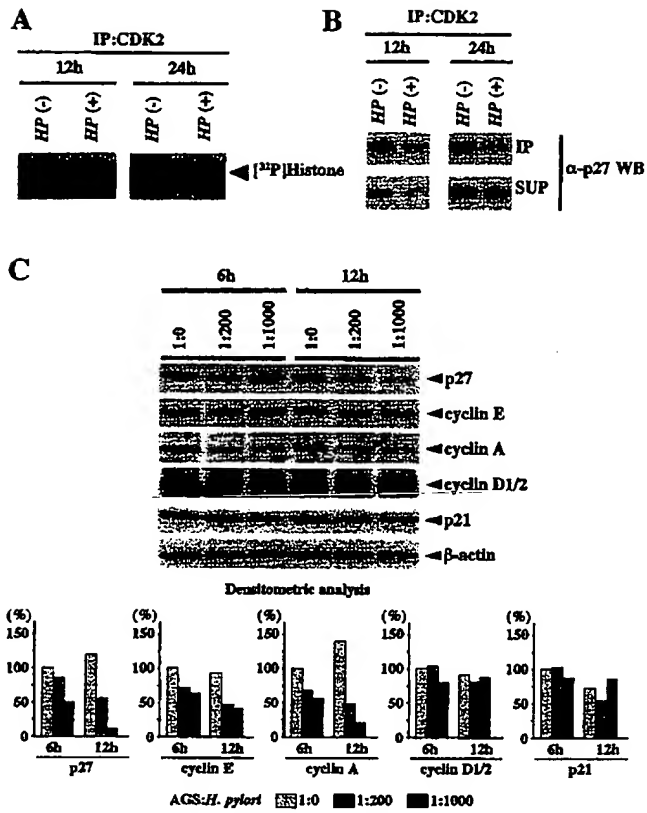

Fig. 5. Effects of *H. pylori* on regulators of the G_1-S transition. AGS cells were serum deprived for 48 h, and then *H. pylori* or serum-free medium only was added, and cells were harvested at the indicated time points. *A*, decrease in CDK2-associated kinase activity by *H. pylori* [*HP* (+)]. *B*, the binding of p27 to CDK2 was not decreased by *H. pylori*. Cell lysates were immunoprecipitated using anti-CDK2 antibody, and immunoprecipitates (*IP*) and 10% aliquots of supernatant (*SUP*) were subjected to electrophoresis. p27 was evaluated by Western blot. *C*, immunoblot demonstrating expression of G_1-S-phase-regulatory proteins in AGS cells. *H. pylori* was added at the indicated AGS: bacterial cell ratios. The *bottom graphs* show the densitometric analysis of protein expression after the addition of *H. pylori*.

Nunc International Corp., Naperville, IL; data not shown). Putative *H. pylori* virulence-associated genes include the *cagA* and *cagE* genes within the *cag* pathogenicity island and the *vacA* gene (37). Incubation of AGS cells with isogenic *H. pylori* strains with loss of function deletions in each of these three genes resulted in a decrease in p27 expression similar to that observed with the wild-type strain (Fig. 9*A*). All of the five clinical strains tested also decreased the level of p27 protein, but strains lacking the entire *cag* island (strains J68, B107, and B166) showed a small decrease in their ability to decrease p27 (Fig. 9*B*), indicating that genes within the *cag* pathogenicity island may play a minor role in decreasing the level of p27 protein. Taken together, these data suggest that the ability of *H. pylori* to reduce p27 is not related to *cagA*, *cage*, or allelotypes of *vacA* or *iceA* but may be partially dependent on other *cag* island genes.

DISCUSSION

Chronic infection with *H. pylori* results in chronic gastritis, which progresses in some susceptible individuals to gastric cancer. Experiments reported here suggest that a pathway that may be relevant to malignant transformation induced by *H. pylori* involves the down-regulation of p27, a cell cycle inhibitor, tumor suppressor, and apoptosis regulator. Previous work from our group (21) and others (22) has demonstrated that chronic *H. pylori* infection is associated with a reduction in the expression of p27 protein in gastric epithelial cells in biopsy specimens, as detected by immunohistochemistry. We now demonstrate that the reduction in levels of p27 protein expression can be reproduced by the coculture of *H. pylori* with gastric epithelial cells and that it is dose and time dependent. Our data also reveal that the decrease in p27 protein is mediated through increased p27 protein breakdown rather than decreased transcription, based on the findings that steady-state p27 mRNA levels were unchanged, whereas pulse-chase analysis demonstrated more rapid protein degradation after the addition of *H. pylori* to gastric epithelial cells.

It is noteworthy that the reduction in p27 that we observed with *H. pylori* occurred independent of *H. pylori*'s effects on the cell cycle. Previous work has established that although chronic *H. pylori* infection *in vivo* is associated with a hyperproliferative state (10), short-term coculture of *H. pylori* with gastric epithelial cells *in vitro* results in an inhibition of cell cycle progression at the G_1-S phase of the cycle (10, 38) and at G_2-M (28). The level of p27 protein is highly cell cycle phase dependent. Therefore we took particular attention in designing experiments that demonstrated that *H. pylori* was capable of down-regulating p27 expression in cells that were not actively proliferating (because p27 expression falls during S phase), thus dissociating the effect of *H. pylori* on p27 expression from the effects of *H. pylori* on cell cycle-dependent changes in p27 expression.

What are the molecular mechanisms responsible for the increased p27 degradation by *H. pylori*? Increased activation of Thr^{187}-dependent ubiquitin-proteasome pathway associated with increased skp2 expression has been reported in several human cancers, including

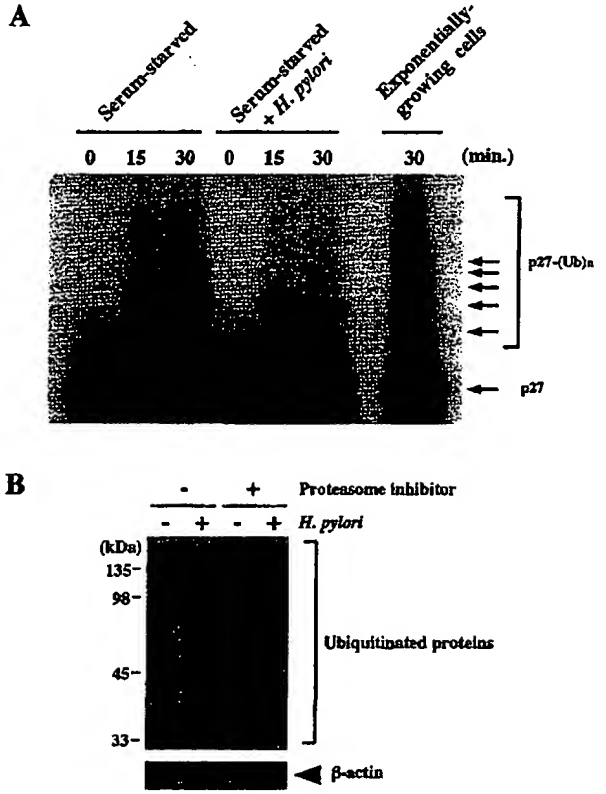

Fig. 6. *H. pylori* decreases protein ubiquitination. *A*, *in vitro* assay of p27 ubiquitination by lysates of AGS cells grown in the absence or presence of *H. pylori*. The incubation time of *in vitro*-translated p27 with the cell lysates is indicated in minutes at the *top*. Mono- and polyubiquitinated p27 species are decreased in lysates from serum-starved AGS cells cocultured with *H. pylori* and increased in lysates from exponentially growing cells. *B*, Western blot analysis of total cell lysates using anti-ubiquitin antibody demonstrating that *H. pylori* decreases ubiquitinated protein expression, detectable when the proteasome activity is inhibited with 50 μM *N*-acetyl-Leu-Leu-norleucinal (ALLN; Sigma Chemical Co.).

colon and gastric cancer (17, 19), and low p27 expression is generally associated with a poor prognosis in cancer (14). These observations are consistent with increased cell proliferation in cancer cells and the demonstration of p27 as a tumor suppressor (16) in an animal model. We demonstrate here that *H. pylori* decreases p27 protein in epithelial cells by up-regulating proteasome-dependent degradation. Our results also suggest that this degradation is associated with neither increased phosphorylation of p27 at Thr^{187}, increased skp2 expression, nor increased p27 ubiquitination. Indeed, the expressions of skp2, Thr^{187}-phosphorylated p27, and ubiquitinated p27 were all decreased by the addition of *H. pylori* to AGS cells. Thus, *H. pylori* increases p27 protein degradation by a mechanism that it is quite different from that responsible for the degradation of p27 during normal cell cycling.

Several alternative pathways that regulate the function and/or expression of p27 protein at a posttranslational level have been described recently, including some that are proteasome independent (33, 39), some that are skp2 independent (34), and some that are Thr^{187} phosphorylation independent (34, 40). Furthermore, in addition to the onset of p27 degradation in late G_1 phase, two groups have reported a separate and distinct p27 degradative pathway active in the G_0-G_1 phase of the cell cycle that is independent of phosphorylation at Thr^{187} (34, 40), although there is disagreement regarding the requirement for skp2 in this pathway. Our data also demonstrate that the reduction of p27 by *H. pylori* is less evident in exponentially growing AGS cells than in serum-starved cells, consistent with *H. pylori* decreasing p27 predominantly or entirely in cells in G_0-G_1 phase, possibly through this Thr^{187}-independent pathway. It is not yet clear whether this more recently discovered pathway is dependent on the phosphorylation of p27 at sites other than Thr^{187}; several other phosphorylated forms of p27 have been described, including p27 phosphorylation downstream of mitogen-activated protein kinase signaling pathways (39) and phosphorylation of serine 10 (41, 42), threonine 157 (43–45), and threonine 198 (by Akt; Ref. 46), which have each been reported to be associated with nuclear export of p27 and reduced p27 protein stability. Whether the apparent abundance of "alternative" p27 degradative pathways, as well as one involving the neddylation of skp2 that obviates the need for p27 phosphorylation before ubiquitination (47), are cell type dependent, cell cycle phase dependent, and stimulus dependent remains to be determined.

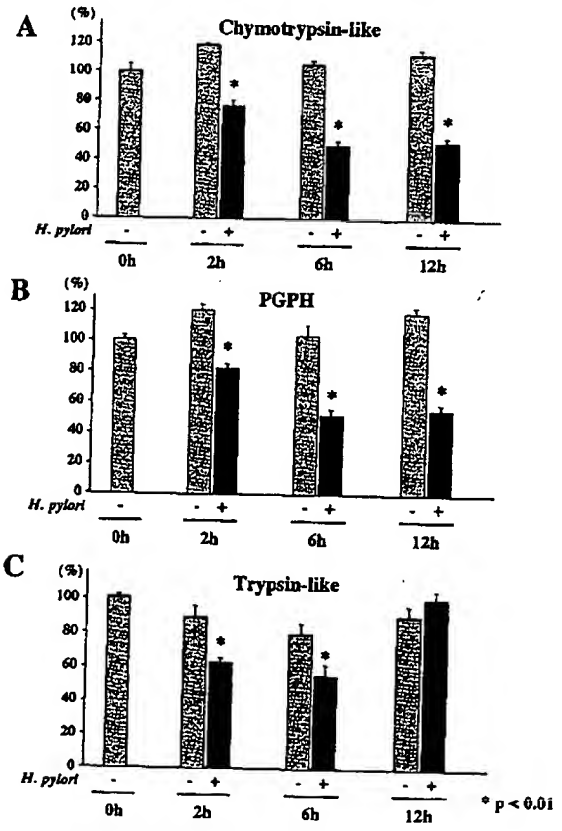

Fig. 7. Proteasomal activities measured *in vitro*. *A*, chymotrypsin-like activity; *B*, peptidylglutamyl peptide hydrolyzing-like activity; *C*, trypsin-like activity. AGS cells were cocultured with *H. pylori* for the indicated times. *Gray columns* show proteasome activity results in the absence of *H. pylori*. All data have been normalized to time 0 (100%). *, $P < 0.01$ by two-way ANOVA with Bonferroni-adjusted *t* tests compared with the absence of *H. pylori*.

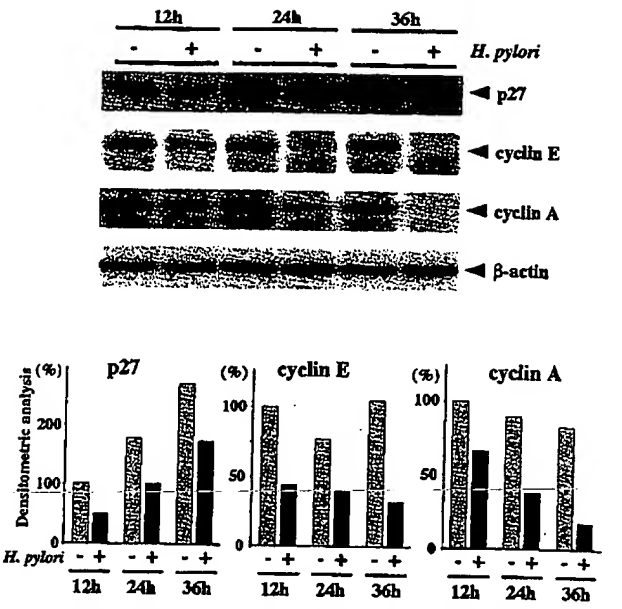

Fig. 8. Effect of *H. pylori* on p27, cyclin E, and cyclin A in MKN28 gastric epithelial cells. *H. pylori* was added to MKN28 cells that had been serum deprived for 48 h. Cells were harvested for immunoblotting at 12, 24, and 36 h. The *bottom graphs* show the densitometric analysis of protein expression.

The above-mentioned mechanisms of increased p27 degradation all converge on ubiquitination as a final common step before proteasome-mediated degradation. However, our data support a proteasome-dependent but ubiquitin-independent degradation pathway. Such a pathway has not previously been described for p27, although it has been described for several other short-lived proteins including $p21^{cip1}$ (48, 49), ornithine decarboxylase (50), and c-Jun (51). We speculate that *H. pylori* may stimulate such a novel proteasome-dependent, ubiquitin-independent pathway of p27 degradation.

Although our results indicate that *H. pylori* regulates the expression of p27 through increased degradation, this may not be the only mechanism responsible for decreasing p27 protein expression during chronic infection by *H. pylori*. In a long-term coculture model during which *H. pylori* selects for apoptosis-resistant gastric epithelial cells, we have documented that the expression of p27 mRNA is reduced in these apoptosis-resistant cells by about 30% by Northern blot (21) and have recently confirmed this result by cDNA microarray analysis.[4] Thus, p27 protein may also be transcriptionally regulated by *H. pylori* during chronic infection.

H. pylori is highly prevalent in human populations and is genetically diverse (37). The ability of *H. pylori* to promote gastric carcinogenesis in only a subset of infected persons is therefore thought to

[4] H. Eguchi, S. Carpentier, S-S. Kim, S. F. Moss. Regulates the apoptotic response of gastric epithelial cells to *Helicobacter pylori*, manuscript in preparation.

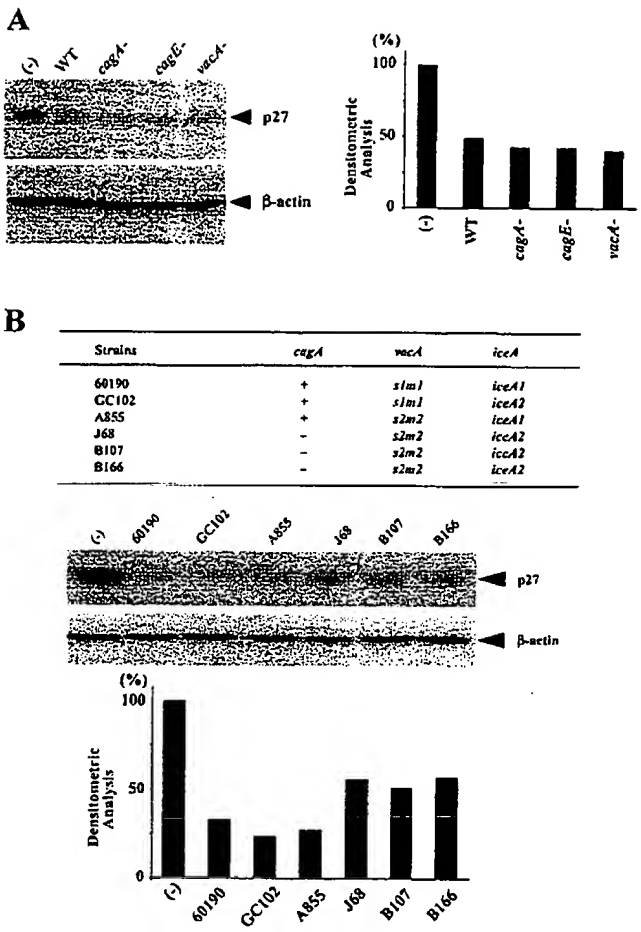

Fig. 9. *A*, AGS cells and wild-type (*WT*) *H. pylori* or its isogenic mutants (*cagA* negative, *cagE* negative, or *vacA* negative) were cocultured for 12 h. Expression of p27 protein was decreased to the same manner by wild-type *H. pylori* or its isogenic mutants. The *right panel* shows the densitometric analysis of p27 expression. *B*, strain 60190 and five clinical strains of *H. pylori* were cocultured for 12 h. *cagA*, *vacA*, and *iceA* status of the strains examined is listed in the *table*. The *bottom graph* shows the densitometric analysis of protein expression. —, no *H. pylori* (control).

be related to both specific bacterial virulence factors and genetically determined host responses to infection, particularly polymorphisms of cytokines and cytokine receptors (52, 53). Bacterial virulence factors with a potential to influence gastric epithelial cell cycling and epithelial cell signal transduction include *H. pylori*'s VacA exotoxin and genes within its *cag* pathogenicity island (10, 28). The *cag* gene products include several that form a type IV bacterial secretory apparatus capable of translocating *H. pylori* products, such as CagA, directly into gastric epithelial cells (54, 55), resulting in the induction of signal transduction pathways of potential relevance to malignant transformation (56). Our results indicate that the reduction of p27 is not dependent on *H. pylori*'s *cagA*, *cagE*, or *vacA* genes, although the requirement for adherence and the results using five clinical strains suggest that other genes within the *cag* pathogenicity island may be responsible for the effects of *H. pylori* on p27. We note with interest the recent report by Sommi *et al.* (57) that *H. pylori* broth culture filtrate may increase the expression of p27, as determined by immunofluorescence. This effect was associated with the inhibition of cell cycle progression in two of the four gastric cell lines examined. However, because *in vivo* p27 expression is decreased in *H. pylori*-infected patients (21, 22), the pathophysiological significance of these findings related to a putative soluble factor secreted by *H. pylori* are currently uncertain. Furthermore, in our experiments, the expression of p27 protein was unchanged when the attachment of *H. pylori* to gastric cells was inhibited by a Transwell membrane, thus indicating that secreted *H. pylori* products are unlikely to be responsible for altering p27. Of interest, this factor studied from broth culture filtrates also did not inhibit cell cycle progression in AGS cells (57), although it is well established that live *H. pylori* bacteria do have this effect (23, 28, 38). Therefore, although these discrepant results may be due to methodological differences, such as the use of highly concentrated broth culture filtrate or the measurement of p27 by flow cytometric analysis rather than immunoblotting, it is conceivable that *H. pylori* does have more than one effect on p27 protein—one that requires attachment, and one that does not.

Regardless of the precise mechanisms by which *H. pylori* alters the expression of the cell cycle inhibitor p27, the functional consequences of decreased p27 are likely to be relevant to gastric carcinogenesis. In an animal model, homozygous or heterozygous loss of p27 causes tissue hyperplasia associated with increased cell proliferation and increased susceptibility to cancer after exposure to exogenous carcinogens (16, 58). Additionally, loss of p27 may reduce cell death through apoptosis (59), thus potentially causing inappropriate and excessive tissue growth.

In summary, our results indicate that *H. pylori* activates pathways leading to the increased degradation of p27 in gastric epithelial cells through a proteasome-dependent, ubiquitin-independent mechanism that is distinct from the physiological pathway of p27 degradation known to be up-regulated in some cancers. Analysis of several *H. pylori* cancer-associated genes suggests that neither *cagA*, *cagE*, nor *vacA* is involved in this process but that other *cag* island genes may play a modest role in the reduction of p27. Further work may define the specific *H. pylori* factors responsible. Regardless of mechanisms, the down-regulation of p27 by *H. pylori* may lead to increased gastric epithelial cell proliferation, decreased apoptosis, and increased risk for gastric cancer.

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